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(57) Abstract

The protein encoded by the human gene *HEC* (highly expressed in cancer) contains a long series of leucine heptad repeats and appears to be crucial for normal mitosis. HEC localizes to the nuclei of interphase cells and redistributes to centromeres during M phase. Ectopic expression of a mutant HEC containing only the heptad repeats results in the inability of cells to divide more than once. Inactivation of HEC results in disordered sister chromatid alignment and separation, as well as in the formation of nonviable cells with multiple, fragmented micronuclei. HEC interacts through its leucine heptad repeats with several proteins involved in mitosis, including nek2, sb1.8, and two different regulatory subunits of the 26S proteasome, MSS1 and p45. These biochemical properties of HEC suggest its potential roles in modulating proteins important for spindle attachment to kinetochores, sister chromatid movement, and M phase progression.

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DESCRIPTION

PROTEINS AND COMPOSITIONS FOR MODULATING MITOSIS

1.0 BACKGROUND OF THE INVENTION

The government owns rights in the present invention pursuant to grant numbers EY05758 and CA58318 from the United States National Institutes of Health.

1.1 Field of the Invention

The invention relates generally to the field of molecular biology and more particularly to compounds and methods comprising novel DNA segments and their encoded polypeptides important in regulation of cell proliferation. The compounds may be adapted to control cell malignancies and various other cell growth abnormalities at the cell mitosis stage.

1.2 Description of the Related Art

In recent years, understanding of cell cycle progression at the molecular level has advanced quickly. When cells are stimulated to divide, *e.g.* by growth factors, a cascade of distinct but intersecting signals transduces the stimulus from cell surface to nucleus. A group of kinases is then activated to initiate events in early G1 phase that soon commit the cell to subsequent phases and to proliferation (Pines, 1995). The role of cyclins, cyclin dependent kinases, and their inhibitors (CKIs) in contributing to the cell cycle machinery has been reviewed (Harper and Elledge, 1996; Pines, 1995).

The ultimate goal of cell division is to assure the high-fidelity transmission of the replicated DNA to daughter cells. The physical separation of pairs of sister chromatids to two daughter cells is orchestrated precisely during M phase, and many of the events involved are highly conserved in all eukaryotes (reviewed by Yanagida, 1995). To ensure faithful progress through cell division, each step is coordinated through a group of structural and regulatory proteins that serve as checkpoints to monitor the timing and accuracy of previous steps (Hartwell, 1992). Mitotic B-type cyclins, cyclin-dependent kinases, other kinases, and components of centromere/kinetochore have all received considerable attention (reviewed by Harper and Elledge, 1996; He *et al.*, 1995). Under normal circumstances, faulty cell cycle events will be sensed and cell cycle progression stalled until the problems with previous steps can be resolved.

In addition to transcriptional control of gene expression and phosphorylation-dephosphorylation, progression of the cell cycle involves the targeted degradation of proteins regulating key transitions points. Two key checkpoints in mitosis, sister chromatid separation and exit to G1, for example, are known to require the destruction of specific proteins such as anaphase inhibitory factors and mitotic cyclins (Glotzer *et al.*, 1991; Holloway *et al.*, 1993; Hunt *et al.*, 1992; Irniger *et al.* 1995). Degradation is primarily accomplished by a pathway that involves ubiquitination (reviewed by Deshaises, 1995), a series of enzymatic modifications that marks proteins for destruction by a multiple-subunit protease called the proteasome. Many components of the ubiquitin pathway and the proteasome have been cloned and characterized in yeast (Hilt and Wolf, 1995) and in humans (Dubiel *et al.*, 1995).

Studies on Mitosis

Progress has recently been made by using fungi, *Drosophila melanogaster*, and *Xenopus laevis* for dissecting molecular and cellular processes during mitosis. Yeasts have been exploited in particular because of the relative ease with which their genes can be manipulated to select mutants defective in mitosis. Several genes that lead to M phase arrest have been isolated (reviewed in Hegemann and Fleig, 1993). Some encode proteins involved in the protein degradation process, *e.g.*, CIM3 (Sug1) and CIM5, both subunits the 26S proteasome in the budding yeast (Ghislain *et al.*, 1993; Swaffield *et al.*, 1992). Some, such as Nuc2 (Hirano *et al.*, 1988) and Cut9 (Samejima and Yanagida, 1994) of fission yeast, are classified by sequences encoding tetratricopeptide repeat (TPR) domains (Goebl and Yanagida, 1991).

Nuc2/CDC27Hs has recently been shown to associate with the centromere and mitotic spindle, and to function in the ubiquitin-mediated protein degradation pathway (King *et al.*, 1995; Tugenreich *et al.*, 1995). Kinases such as NimA in *Aspergillus nidulans* (Osmani *et al.*, 1988) are homologous to human Nek2 (Schultz and Nigg, 1993) and phosphatases such as protein phosphatase type 1-α or PP1a, (Booher and Beach, 1989; Doonan and Morris, 1989; Cyert and Thorner, 1989) also lead to mitotic arrest when inactivated. Other proteins such as SMC1 and SMC2 are essential for chromosome segregation and condensation (Strunnikov *et al.*, 1993; 1995) or, such as tubulin (Weisenberg and Rosenfeld, 1975) and kinesin-like proteins (reviewed in Walczak and Mitchison, 1996), for spindle formation. Although the

number of known proteins and genes required for chromatid separation is rapidly increasing, the precise mechanisms responsible for mitotic molecular events remain elusive. Recent evidence suggests that the metaphase arrest induced by primary structural abnormalities in the kinetochore may also require interaction with proteins involved in spindle assembly and in monitoring of mitotic checkpoints (Wang and Burke, 1995; Wells and Murray, 1996).

Proteins with properties similar to those of HEC have been characterized. The nuclear protein that associates with the mitotic apparatus (NuMA), for example, is also required for the proper completion of mitosis (Compton and Cleveland, 1993). When NuMA is inactivated, either by strategic mutation or by microinjection of anti-NuMA antibodies prior to mitosis, abnormalities in chromosome alignment and segregation result in the formation of daughter cells with micronuclei (Compton and Cleveland, 1993; Compton and Luo, 1995; Gaglio *et al.*, 1995; Kallijoki *et al.*, 1993; Yang *et al.*, 1992; Zeng *et al.*, 1994). Furthermore, several features of the abnormal mitotic phenotype such as multiple spindle poles and disordered metaphase chromosome alignment--are observed in mammalian cells treated with drugs like taxol and vinca alkaloids that directly disrupt microtubule structure (Jordon *et al.*, 1992; 1993; Tinwell and Ashby, 1991). These drugs all arrest cells in M phase, as does the injection of neutralizing antibodies to all known CENPs.

Investigation of the molecules and checkpoints involved in chromatid segregation and checkpoint control is important for aneuploidy, or alteration of chromosome number, is common in cancer cells and apparently results from improper chromosomal segregation in M phase (Solomon *et al.*, 1991). The strong association of aneuploidy with cancer suggests that altered regulation of the mitotic process also contributes substantially to oncogenesis and to tumor progression. In addition, defects of the ubiquitin-mediated proteolytic pathway may enhance genomic instability or cause loss of control of cell growth and proliferation by affecting degradation of cyclins or CKIs (Bai *et al.*, 1996; Zhang *et al.*, 1995). For example, the removal of mitotic checkpoints while preserving daughter cell viability could confer clonal growth advantages and lead eventually to cancer.

Thus an understanding of the molecular events of mitosis is important in learning how to control the chromosomal abnormalities observed in malignant cells might originate. This will allow identification and development of agents to control cell proliferation. Specific

proteins crucial for mitotic progression must be identified, characterized, and linked to known pathways of mitotic protein regulation.

2.0 SUMMARY OF THE INVENTION

The inventors describe the characterization of a human nuclear protein, HEC, which was isolated as clone 15 (C15) through its interaction with the retiniblastoma protein (Rb) in the yeast-two hybrid system (Durfee *et al.*, 1993). HEC protein appears to play an important role in chromosome segregation during M phase, since it is expressed most abundantly in rapidly dividing cells, localizes to centromeres during mitosis, and when inactivated leads to severe abnormalities in chromosome congression and segregation which prevent subsequent cell divisions.

The inventors have characterized a novel gene, *HEC* , which clearly plays a critical role in M phase. Several lines of evidence indicate that HEC serves as a regulator to coordinate sister chromatid segregation. First, HEC is expressed most abundantly in mitotic cells but not in those that have terminally differentiated. Second, it redistributes to the centromeres of dividing cells. Third, inactivation of HEC by microinjection with specific antibodies severely disturbs M phase, as does expression of a dominant-negative HEC mutant containing only the long series of leucine heptad repeats. Finally, HEC was shown to interact, through its leucine heptad repeat domain, with several proteins important for mitosis, including Nek2, sb1.8, and two different regulatory subunits of the 26S proteasome, MSS1 and p45. These results indicate that HEC may function to regulate proteins mediating spindle attachments to kinetochores and to modulate checkpoints for M phase progression.

The data suggest that HEC may function as an "adaptor molecule" through its long leucine heptad repeats. In this respect, HEC may have properties similar to those of the budding yeast Skp1 protein (Bai *et al.*, 1996; Connelly and Heiter, 1996): it may alter the conformation of multiple-subunit complexes and bring together a number of proteins, including components of the mitotic spindle or kinetochore, components of the 26S proteasome, kinases or phosphatases, and checkpoint monitors. The dynamics of the spindle apparatus are modulated, at least in part, by the same kinases and components of the proteasome and ubiquitin-dependent protein degradation pathway with which HEC seems to interact (Holloway *et al.*, 1993; Irniger *et al.*, 1995; King *et al.*, 1995; Tugenreich *et al.*, 1995).

The regulatory events during chromosome alignment and separation are rapid and precisely timed, and they can be profoundly disturbed without a coordinating molecule such as HEC.

2.1 Novel Mitosis-Regulating Polypeptides

In an important aspect therefore, the present invention relates to the discovery of a novel human nuclear protein found to be highly expressed in cancer cells. The new protein, HEC, appears to be important in mitosis, possibly in regulation of normal progression of M phase. The peptide sequence (SEQ ID NO:2) has little homology with other Genbank database deposited protein sequences available at the time of the invention.

2.2 HEC Pharmaceutical Compositions

Another aspect of the present invention includes novel compositions comprising isolated and purified HEC proteins or nucleic acids which encode HEC protein. It will, of course, be understood that one or more than one HEC genes may be used in the methods and compositions of the invention. The nucleic acid delivery methods may thus entail the administration of one, two, three, or more, homologous genes. The maximum number of genes that may be applied is limited only by practical considerations, such as the effort involved in simultaneously preparing a large number of gene constructs or even the possibility of eliciting an adverse cytotoxic effect.

Compositions employing the novel HEC proteins will contain a biologically effective amount of the peptide or peptides. As used herein a "biologically effective amount" of a peptide or composition refers to an amount effective to alter or modulate M phase mitosis. As disclosed herein, different peptide amounts may be effective, as shown *in vitro* and *in vivo* such as those between about 6 to about 11 mg/kg.

Clinical doses will of course be determined by the nutritional status, age, weight and health of the patient. The quantity and volume of the peptide composition administered will depend on the subject and the route of administration. The precise amounts of active peptide required will depend on the judgment of the practitioner and may be peculiar to each individual. However, in light of the data presented herein, the determination of a suitable dosage range for use in humans will be straightforward.

The compositions that provide HEC in accordance with the present invention will be compositions that contain the full length peptide which has about 633 amino acid residues and a

molecular weight of about 76 kDa or functional fragments and variants thereof such as the sequence represented by SEQ ID NO: 2 or the region between amino acids 254 and 621 of SEQ ID NO:2. The term "a peptide" or "a polypeptide" in this sense means at least one peptide or polypeptide which includes a sequence of any of the aforementioned structures or variants thereof. The terms peptide and polypeptide are used interchangeably.

In addition to including an amino acid sequence in accordance with SEQ ID NO:2, the peptides may include various other shorter or longer fragments or other short peptidyl sequences of various amino acids. In certain embodiments, the peptides may include a repeat of shorter sequences, for example, the leucine-repeat heptad region between amino acids 254 and 621 of SEQ ID NO:2, or additional sequences such as short targeting sequences, tags, labeled residues, amino acids contemplated to increase the half life or stability of the peptide or any additional residue for a designated purpose, so long as the peptide still functions to regulate mitosis. Such functionality may be readily determined by assays such as those described herein.

Any of the commonly occurring amino acids may be incorporated into the peptides, including alanine, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. Likewise, any of the so-called rare or modified amino acids may also be incorporated into a peptide of the invention, including: 2-Aminoadipic acid, 3-Aminoadipic acid, beta-Alanine (beta-Aminopropionic acid), 2-Aminobutyric acid, 4-Aminobutyric acid (piperidinic acid), 6-Aminocaproic acid, 2-Aminoheptanoic acid, 2-Aminoisobutyric acid, 3-Aminoisobutyric acid, 2-Aminopimelic acid, 2,4-Diaminobutyric acid, Desmosine, 2,2'-Diaminopimelic acid, 2,3-Diaminopropionic acid, N-Ethylglycine, N-Ethylasparagine, Hydroxylysine, allo-Hydroxylysine, 3-Hydroxyproline, 4-Hydroxyproline, Isoeesmosine, allo-Isoleucine, N-Methylglycine sarcosine), N-Methylisoleucine, N-Methylvaline, Norvaline, Norleucine and Ornithine.

The compositions of the invention may include a peptide modified to render it biologically protected. Biologically protected peptides have certain advantages over unprotected peptides when administered to human subjects and, as disclosed in U.S. patent 5,028,592, incorporated herein by reference, protected peptides often exhibit increased pharmacological activity.

Compositions for use in the present invention may also comprise peptides which include all L-amino acids, all D-amino acids or a mixture thereof. The use of D-amino acids may confer additional resistance to proteases naturally found within the human body and are less immunogenic and can therefore be expected to have longer biological half lives.

Likewise, compositions that make use of HEC-encoding genes are also contemplated. The particular combination of genes may be two or more variants of *hec* genes; or it may be such that a HEC protein gene is combined with another gene and/or another protein such as a Nuc2, Cut9, NimA, Nek2 or phosphatases such as protein phosphatase 1-α or PP1 may even be combined with a gene encoding a cell surface receptor capable of interacting with the polypeptide product of the first gene.

In using multiple genes, they may be combined on a single genetic construct under control of one or more promoters, or they may be prepared as separate constructs of the same or different types. Thus, an almost endless combination of different genes and genetic constructs may be employed. Certain gene combinations may be designed to, or their use may otherwise result in, achieving synergistic effects on cell growth and/or stimulation of an immune response. Any and all such combinations are intended to fall within the scope of the present invention. Indeed, many synergistic effects have been described in the scientific literature, so that one of ordinary skill in the art would readily be able to identify likely synergistic gene combinations, or even gene-protein combinations.

It will also be understood that, if desired, the nucleic acid segment or gene encoding a HEC polypeptide could be administered in combination with additional agents, such as, *e.g.*, proteins or polypeptides or various pharmaceutically active agents. So long as the composition comprises a HEC gene, there is virtually no limit to other components which may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The nucleic acids may thus be delivered along with various other agents as required in the particular instance.

Pharmaceutical compositions prepared in accordance with the present invention find use in several applications, including inhibition or modulation of proliferation of malignant cells or regulation of normal cell proliferation. Such methods generally involve administering to a mammal a pharmaceutical composition comprising an immunologically effective amount of a HEC composition. This composition may include an immunologically-effective amount of

either a HEC peptide or a HEC-encoding nucleic acid composition. Such compositions may also be used to generate an immune response in a mammal.

Therapeutic kits comprising HEC peptides or HEC-encoding nucleic acid segments comprise another aspect of the present invention. Such kits will generally contain, in suitable container means, a pharmaceutically acceptable formulation of HEC peptide or a HEC-encoding nucleic acid composition. The kit may have a single container means that contains the HEC composition or it may have distinct container means for the HEC composition and other reagents which may be included within such kits.

The components of the kit may be provided as liquid solution(s), or as dried powder(s). When the components are provided in a liquid solution, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

In related embodiments, the present invention contemplates the preparation of diagnostic kits that may be employed to detect the presence of HEC proteins or peptides and/or antibodies in a sample. Generally speaking, kits in accordance with the present invention will include a suitable HEC protein or peptide or antibody directed against such a protein or peptide, together with an immunodetection reagent and a means for containing the antibody or antigen and reagent. The components of the diagnostic kits may be packaged either in aqueous media or in lyophilized form.

The immunodetection reagent will typically comprise a label associated with the antibody or antigen, or associated with a secondary binding ligand. Exemplary ligands might include a secondary antibody directed against the first antibody or antigen or a biotin or avidin (or streptavidin) ligand having an associated label. Of course, as noted above, a number of exemplary labels are known in the art and all such labels may be employed in connection with the present invention. The kits may contain antibody-label conjugates either in fully conjugated form, in the form of intermediates, or as separate moieties to be conjugated by the user of the kit.

The container means will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the antigen or antibody may be placed, and

preferably suitably aliquoted. Where a second binding ligand is provided, the kit will also generally contain a second vial or other container into which this ligand or antibody may be placed. The kits of the present invention will also typically include a means for containing the antibody, antigen, and reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

2.3 HEC Antibodies

In another aspect, the present invention includes one or more antibodies that are immunoreactive with a polypeptide of the invention. An antibody can be a polyclonal or a monoclonal antibody. In a preferred embodiment, an antibody is a monoclonal antibody, as illustrated with antibody 9G3 which is specific for HEC as shown in immunoprecipitation and immunoblotting studies. Polyclonal anti-serum such as polyclonal anti-C15 serum is also part of the invention. This polyclonal anti-serum recognizes the HEC protein that has the amino acid sequence of SEQ ID NO:2. Means for preparing and characterizing antibodies are well known in the art (See, *e.g.*, Howell and Lane, 1988).

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster or a guinea pig. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Antibodies, both polyclonal and monoclonal, specific for HEC or selected epitopes of HEC, may be prepared using conventional immunization techniques, as will be generally known to those of skill in the art. A composition containing antigenic epitopes of HEC can be used to immunize one or more experimental animals, such as a rabbit or mouse, which will then proceed to produce specific antibodies against HEC. Polyclonal antisera may be obtained, after allowing time for antibody generation, simply by bleeding the animal and preparing serum samples from the whole blood.

To obtain monoclonal antibodies, one initially immunizes an experimental animal, often preferably a mouse, with a LCRF composition. One then, after a period of time sufficient to

allow antibody generation, obtains a population of spleen or lymph cells from the animal. The spleen or lymph cells are then be fused with cell lines, such as human or mouse myeloma strains, to produce antibody-secreting hybridomas. These hybridomas may be isolated to obtain individual clones which can then be screened for production of antibody to the desired HEC peptide.

Following immunization, spleen cells are removed and fused, using a standard fusion protocol with plasmacytoma cells to produce hybridomas secreting monoclonal antibodies against HEC. Hybridomas which produce monoclonal antibodies to the selected antigens are identified using standard techniques, such as ELISA and Western blot methods. Hybridoma clones can then be cultured in liquid media and the culture supernatants purified to provide the HEC-specific monoclonal antibodies.

It is proposed that the monoclonal antibodies of the present invention will find useful application in standard immunochemical procedures, such as ELISA and Western blot methods, as well as other procedures which may utilize antibody specific to HEC epitopes.

Additionally, it is proposed that monoclonal antibodies specific to the particular mitosis regulating protein may be utilized in other useful applications. For example, their use in immunoabsorbent protocols may be useful in purifying native or recombinant HEC species or variants thereof.

In general, both poly- and monoclonal antibodies against HEC may be used in a variety of embodiments. For example, they may be employed in antibody cloning protocols to obtain cDNAs or genes encoding HEC or related proteins. They may also be used in inhibition studies to analyze the effects of HEC in cells or animals. Anti-HEC antibodies will also be useful in immunolocalization studies to analyze the distribution of HEC during various cellular events, for example, to determine the cellular or tissue-specific distribution of the HEC peptide under different physiological conditions. A particularly useful application of such antibodies is in purifying native or recombinant HEC, for example, using an antibody affinity column. The operation of all such immunological techniques will be known to those of skill in the art in light of the present disclosure.

HEC mRNA is Expressed Abundantly in Rapidly Dividing Cancer Cells

3.0 BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. *HEC* mRNA expression.

FIG. 1*A*. Northern blot analysis of poly A selected RNA (2 μg each) from human brain (lane 1) and WERI-RB-27 cells (lane 2), probed with a 1.8 kb fragment of the *HEC* cDNA clone.

FIG 1B. Northern blot analysis of total RNA from twelve different sources: 1, CV1 monkey kidney cells; 2, human brain; 3, C4-I cervical carcinoma; 4, C4-II cervical carcinoma; 5, MS751 cervical carcinoma; 6, SiHa cervical carcinoma; 7, Caski cervical carcinoma; 8, Molt4 acute lymphocytic leukemia; 9, T47D breast carcinoma; 10, HT-3 cervical carcinoma; 11, SW620 colon carcinoma; 12, WERI-RB-27 retinoblastoma. The blot was probed with C15 and with Gβ-like cDNA, respectively. Gb-like mRNA is expressed constitutively and therefore served as an internal control. The amounts of *HEC* mRNA relative to Gβ-like mRNA were determined by densitometry of the RNA blots.

FIG. 1C. *HEC* mRNA expression varies with progression of the cell cycle. CV1 monkey kidney cells were arrested at various stages of the cell cycle by serum deprivation or drug treatment. Lanes: 1, G1 (density arrest, time 0); 2, late G1 (8 hr after release from density arrest); 3, G1/S boundary (aphidicolin arrest); 4, S (4 hr after release from aphidicolin arrest); 5, M (nocodazole arrest). E2F-1 mRNA expression, which peaks at G1/S, and Gβ-like mRNA expression served as internal controls.

FIG. 2. HEC cDNA sequence and its encoded protein.

FIG. 2A. The complete nucleotide sequence of HEC cDNA. A potential NimA phosphorylation site (Ser 165) is underlined and a long leucine heptad repeat sequence is marked with a series of circled residues.

FIG. 2B. A protein with an apparent molecular mass of 76 kD was identified specifically by polyclonal anti-HEC serum. Mouse sera raised against an GST-C15 fusion protein were used to immunoprecipitate ^{35}S-methionine labeled proteins; either from the *in vitro*

translated, full-length *HEC* cDNA (lanes 1-3), or from metabolically labeled T24 bladder carcinoma cells (lanes 4-6). For lanes 2 and 4, preimmune serum was used rather than anti-C15 antibodies. In lane 6, anti-C15 antibodies were preabsorbed with the GST-C15 antigen before immunoprecipitation.

FIG. 3. HEC distribution in organs, rapidly dividing cells, and differentiating cells.

FIG. 3A. HEC protein expression in whole mouse organs. HEC immunoprecipitated from organ lysates was detected in thymus, spleen, testis, and ovary + uterus. p84 served as a loading control.

FIG. 3B. HEC expression peaks at M phase. T24 cells were either unsynchronized (lane 1), or synchronized at G1 and released for various periods of time (G8 = 8 hours after release, *etc.*). Hypophosphorylated Rb protein ($p110^{RB}$) and various phosphorylated forms ($pp110^{RB}$) marked stages of the cell cycle: G1 (lanes 2-5); G1/S boundary (lane 6); S (lane 7); and M (lane 8). P84 again served as an internal control for protein loading.

FIG. 3C. U937 lymphoma cells in the exponential phase of proliferation were induced by the addition of phorbol ester (TPA) to differentiate. In rapidly dividing cells at time 0, Rb protein exists primarily in hyperphosphorylated states ($pp110^{RB}$); after cell cycle arrest and terminal differentiation to monocytes/macrophages at 96 hours, Rb is primarily hypophosphorylated ($p110^{RB}$). In contrast, HEC is present in proliferating cells but not in terminally differentiated cells.

FIG. 3D. Unsynchronized (U) NIH 3T3-L1 preadipocytes, identical cells synchronized at G1/G0 by density arrest (time 0), and cells induced to differentiate terminally to adipocytes by hormonal treatment (time 1-6 days after treatment) were analyzed in a manner similar to that used in *C*.

FIG. 4. Subcellular localization of HEC.

FIG. 4A. Biochemical fractionation of T24 cells (T) into nuclear (N), cytoplasmic (C), and membrane (M) components. Each fraction was immunoprecipitated by either anti-C15 antibodies or 11D7 anti-Rb mAb which detected Rb from the same cells as a marker for nuclear protein. The same subcellular fractions were also incubated with glutathione agarose beads to identify glutathione-S-transferase, which served as a marker for cytoplasmic proteins.

FIG. 4B. Immunocytochemical localization during different phases of the cell cycle. Panels: *a*, T24 cells fixed in late G1 phase show scanty staining in nuclei (original

magnification 400x); *b*, cells at the G1/S boundary stain more strongly in nuclei and in perinuclear cytoplasm; *c*, a cell in S phase; *d*, a cell in anaphase (higher magnification, 1000 ×) showing staining surrounding the entire cell and more discrete staining in paired dots that are moving away from the center.

FIG. 4C Metaphase chromosomes were first stained with DAPI. The same microscopic field was then analyzed after indirect immunofluorescence antibody staining. Panels: *a*, anti-C15 polyclonal serum (1:1000 dilution) and FITC-tagged anti-mouse IgG secondary antibodies localize HEC to centromeres; *b*, human autoimmune (CREST) antiserum, which recognizes centromere proteins, and Texas Red-tagged secondary antibodies also labeled centromeres; *c*, digital overlay of anti-C15 and CREST antiserum images.

FIG. 5. Expression of HEC deletion mutant interferes with mitosis.

FIG. 5A, Full-length HEC, GFP-15PA containing only amino acids 1-250, and GFP-15Pst encoding amino acids 251-618 of the entire leucine heptad repeat domain.

FIG. 5B. Detection of GFP and GFP-HEC fusion proteins in transfected Saos-2 cells. After transient transfection, cell lysates were separated by SDS-PAGE. Expression of GFP fusion proteins was determined by immunoprecipitation with an anti-Myc1-9E10 mAb (Evan, *et al.*, 1985), followed by blotting with anti-GFP antibody (Clonetech. Palo Alto, CA). Asterisks mark GFP (lane 2), GFP-15PA (lane 3), and GFP-15Pst (lane 4) fusion proteins. Arrow marks the IgG heavy chain.

FIG. 5C. Localization of GFP and GFP-HEC fusion proteins in Saos-2 cells. DAPI (blue, *a*, d, *g*) identifies DNA in nuclei; GFP autofluorescence (green, *b*, *e*, *h*) shows the subcellular location of the various GFP-HEC fusion proteins; and indirect immunofluorescence with anti-α-tubulin primary antibody and Texas Red-labeled secondary antibodies marks the location of a-tubulin (*c*, *f*, *i*).

FIG. 6. Division of Saos-2 cells ectopically expressing GFP-HEC fusion proteins. Cells expressing GFP alone or GFP-15PA divide to form 2-and 4-cell colonies. Cells expressing GFP-15Pst, however, are unable to complete division more than once; they form few 2-cell colonies and no 4-cell colonies during the 99 hour observation period.

FIG. 7. Microinjection of anti-HEC results in aberrant mitosis.

FIG. 7A. Characterization of mouse monoclonal antibody 9G3. The antibody was generated against the same antigen used to make polyclonal anti-C15 and used for straight immunoblotting of protein lysates from 5×10^5 CV1 (lane 1) or T24 cells (lane 2).

FIG. 7B. T24 cells were released from density arrest and allowed to proceed through the cell cycle. Twenty-four hours after release, the majority of cells were in S phase, at which time they were microinjected with either nonspecific mouse IgG (panels *a*, *b*) or mAb 9G3 (panels *c*, *d*). Twenty-six hours later, after they had passed through mitosis, cells were fixed and analyzed by indirect immunofluorescence staining. Panels: *a*, *c*, DAPI fluorescence; *b*, *d*, staining with anti-mouse IgG antibodies. The arrowheads in each panel identify the daughters of cells successfully microinjected. The daughter cells marked by arrows in panels *a* and *c* were not microinjected.

FIG 7C. Cells at different phases of mitosis. Panels *a-f* show normal mitosis in uninjected cells and cells microinjected with control mouse IgG; panels *g-l* show mitotic phases of cells injected with anti-HEC mAb 9G3. Blue fluorescence is from DAPI, red fluorescence from rabbit anti-tubulin primary antibody and Texas Red conjugated anti-rabbit IgG secondary antibody. Panels: *a*, *b*, prophase; *c*, *d*, metaphase; *e*, *f*, early telophase; *g*, *h*, abnormal spindle formation with at least four discrete spindle poles; *i*, *j*, disordered chromatid alignment and absence of a distinct metaphase plate; *k*, *l*, abnormal chromatid segregation: the chromatids in *k* align along a nearly horizontal axis but the corresponding spindles in *l* pull in a direction 90° opposite.

4.0 DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Abnormalities observed during metaphase and anaphase can result from primary problems earlier in the cell cycle. Microinjection of polyclonal CREST serum, which recognizes several different centromeric proteins, for example, is known to disrupt kinetochore assembly and block progression through mitosis (Bernat *et al.*, 1990; Simerly *et al.*, 1990). The timing of antiserum injection in these studies was crucial. If injected into the cytoplasm or nucleus during S or G2 phases, anti-centromere antibodies caused abnormalities in mitosis very similar to those described here after injection of specific anti-HEC antibodies. In contrast, if injected into nuclei after alignment of metaphase chromatids had been completed,

anti-centromere antibodies had little effect on the subsequent progression of mitosis (Bernat *et al.*, 1990).

In the present work, anti-HEC antibodies were microinjected into cells during S phase, and nuclear morphology was determined at a time point 26 hours later when all cells should have completed mitosis. The inventors cannot exclude prolongation of M phase in cells injected with mAb 9G3, but cells fixed after the completion of abnormal mitosis nonetheless underwent karyokinesis and cytokinesis. In normal cells, "wait anaphase" checkpoints sense tension and kinetochore attachments to microtubules (reviewed in Pluta *et al.*, 1995). These checkpoints normally delay or prevent completion of mitosis in cells with inaccurate or incomplete division of chromosomes to daughter cells (Pluta *et al.*, 1995; Rieder and Salmon 1994). In anti-HEC-injected cells, such checkpoints appear to be partially or completely bypassed. This finding indicated that HEC may have roles other than those directly related to spindle attachment at the centromere. Abnormal conditions in the spindle apparatus, whose morphology probably dictates the location of the cleavage furrow during cytokinesis (Bernat *et al.*, 1990), might explain the bypass of normal checkpoints.

In contrast, HEC inactivation does not arrest cells in mitosis, but allows them to proceed aberrantly. This observation implies a problem with checkpoint control in cells in which HEC has been inactivated.

HEC may function as an adaptor to modulate the ubiquitin-dependent proteolysis machinery, centromere attachments, spindle movement, and checkpoint proteins. While the detailed mechanism by which HEC functions prior to and during mitosis is not fully determined, HEC's location at the centromere/kinetochore indicates that it may be involved in spindle attachment to chromosomes during prophase, and indirectly in subsequent chromosome movement. The lack of a signature tubulin-binding domain in the HEC molecule, however, argues against direct microtubule attachment. The associations of HEC with a mitosis-specific kinase and with several subunits of the proteasome suggest other potential ways by which HEC may influence chromosome congression, separation, or segregation.

The present work indicates that many of the HEC-associated proteins isolated by the yeast two-hybrid screen bind *in vitro* to different and distinct regions within the long leucine heptad repeat domain of the HEC protein. Both MSS1 and Nek2 co-immunoprecipitate with

HEC specifically in late S or M phases. These data suggest that the interaction between HEC and other mitotic proteins is likely to be biologically significant in mammalian cells.

4.1 ELISAs

ELISAs may be used in conjunction with the invention. In an ELISA assay, proteins or peptides incorporating HEC antigenic sequences are immobilized onto a selected surface, preferably a surface exhibiting a protein affinity such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed material, it is desirable to bind or coat the assay plate wells with a nonspecific protein that is known to be antigenically neutral with regard to the test antisera such as bovine serum albumin (BSA), casein or solutions of powdered milk. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

After binding of antigenic material to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the antisera or clinical or biological extract to be tested in a manner conducive to immune complex (antigen/antibody) formation. Such conditions preferably include diluting the antisera with diluents such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween®. These added agents also tend to assist in the reduction of nonspecific background. The layered antisera is then allowed to incubate for from about 2 to about 4 hr, at temperatures preferably on the order of about 25° to about 27°C. Following incubation, the antisera-contacted surface is washed so as to remove non-immunocomplexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween®, or borate buffer.

Following formation of specific immunocomplexes between the test sample and the bound antigen, and subsequent washing, the occurrence and even amount of immunocomplex formation may be determined by subjecting same to a second antibody having specificity for the first. To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the antisera-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor

the development of immunocomplex formation (*e.g.*, incubation for 2 hr at room temperature in a PBS-containing solution such as PBS/Tween®).

After incubation with the second enzyme-tagged antibody, and subsequent to washing to remove unbound material, the amount of label is quantified by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline)-6-sulfonic acid (ABTS) and H_2O_2, in the case of peroxidase as the enzyme label. Quantitation is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectrum spectrophotometer.

4.2 Epitopic Core Sequences

The present invention is also directed to protein or peptide compositions, free from total cells and other peptides, which comprise a purified protein or peptide which incorporates an epitope that is immunologically cross-reactive with one or more anti-HEC antibodies.

As used herein, the term "incorporating an epitope(s) that is immunologically cross-reactive with one or more anti-HEC antibodies" is intended to refer to a peptide or protein antigen which includes a primary, secondary or tertiary structure similar to an epitope located within a HEC polypeptide. The level of similarity will generally be to such a degree that monoclonal or polyclonal antibodies directed against the HEC polypeptide will also bind to, react with, or otherwise recognize, the cross-reactive peptide or protein antigen. Various immunoassay methods may be employed in conjunction with such antibodies, such as, for example, Western blotting, ELISA, RIA, and the like, all of which are known to those of skill in the art.

The identification of HEC epitopes, and/or their functional equivalents, suitable for use in vaccines is a relatively straightforward matter. For example, one may employ the methods of Hopp, as taught in U.S. Patent 4,554,101, incorporated herein by reference, which teaches the identification and preparation of epitopes from amino acid sequences on the basis of hydrophilicity. The methods described in several other papers, and software programs based thereon, can also be used to identify epitopic core sequences (see, for example, Jameson and Wolf, 1988; Wolf *et al.*, 1988; U.S. Patent Number 4,554,101). The amino acid sequence of these "epitopic core sequences" may then be readily incorporated into peptides, either through the application of peptide synthesis or recombinant technology.

Preferred peptides for use in accordance with the present invention will generally be on the order of about 5 to about 25 amino acids in length, and more preferably about 8 to about 20 amino acids in length. It is proposed that shorter antigenic HEC-derived peptide sequences will provide advantages in certain circumstances, for example, in the preparation of vaccines or in immunologic detection assays. Exemplary advantages include the ease of preparation and purification, the relatively low cost and improved reproducibility of production, and advantageous biodistribution.

It is proposed that particular advantages of the present invention may be realized through the preparation of synthetic peptides which include modified and/or extended epitopic/immunogenic core sequences which result in a "universal" epitopic peptide directed to HEC and HEC-related sequences. It is proposed that these regions represent those which are most likely to promote T-cell or B-cell stimulation in an animal, and, hence, elicit specific antibody production in such an animal.

An epitopic core sequence, as used herein, is a relatively short stretch of amino acids that is "complementary" to, and therefore will bind, antigen binding sites on transferring-binding protein antibodies. Additionally or alternatively, an epitopic core sequence is one that will elicit antibodies that are cross-reactive with antibodies directed against the peptide compositions of the present invention. It will be understood that in the context of the present disclosure, the term "complementary" refers to amino acids or peptides that exhibit an attractive force towards each other. Thus, certain epitope core sequences of the present invention may be operationally defined in terms of their ability to compete with or perhaps displace the binding of the desired protein antigen with the corresponding protein-directed antisera.

In general, the size of the polypeptide antigen is not believed to be particularly crucial, so long as it is at least large enough to carry the identified core sequence or sequences. The smallest useful core sequence anticipated by the present disclosure would generally be on the order of about 5 amino acids in length, with sequences on the order of 8 or 25 being more preferred. Thus, this size will generally correspond to the smallest peptide antigens prepared in accordance with the invention. However, the size of the antigen may be larger where desired, so long as it contains a basic epitopic core sequence.

The identification of epitopic core sequences is known to those of skill in the art, for example, as described in U.S. Patent 4,554,101, incorporated herein by reference, which teaches

the identification and preparation of epitopes from amino acid sequences on the basis of hydrophilicity. Moreover, numerous computer programs are available for use in predicting antigenic portions of proteins (see *e.g.*, Jameson and Wolf, 1988; Wolf *et al.*, 1988). Computerized peptide sequence analysis programs (*e.g.*, DNAStar® software, DNAStar, Inc., Madison, Wisc.) may also be useful in designing synthetic HEC peptides and peptide analogs in accordance with the present disclosure.

Syntheses of epitopic sequences, or peptides which include an antigenic epitope within their sequence, are readily achieved using conventional synthetic techniques such as the solid phase method (*e.g.*, through the use of commercially available peptide synthesizer such as an Applied Biosystems Model 430A Peptide Synthesizer). Peptide antigens synthesized in this manner may then be aliquoted in predetermined amounts and stored in conventional manners, such as in aqueous solutions or, even more preferably, in a powder or lyophilized state pending use.

In general, due to the relative stability of peptides, they may be readily stored in aqueous solutions for fairly long periods of time if desired, *e.g.*, up to six months or more, in virtually any aqueous solution without appreciable degradation or loss of antigenic activity. However, where extended aqueous storage is contemplated it will generally be desirable to include agents including buffers such as Tris or phosphate buffers to maintain a pH of about 7.0 to about 7.5. Moreover, it may be desirable to include agents which will inhibit microbial growth, such as sodium azide or Merthiolate. For extended storage in an aqueous state it will be desirable to store the solutions at 4°C, or more preferably, frozen. Of course, where the peptides are stored in a lyophilized or powdered state, they may be stored virtually indefinitely, *e.g.*, in metered aliquots that may be rehydrated with a predetermined amount of water (preferably distilled) or buffer prior to use.

4.3 Immunoprecipitation

The antibodies of the present invention are particularly useful for the isolation of antigens by immunoprecipitation. Immunoprecipitation involves the separation of the target antigen component from a complex mixture, and is used to discriminate or isolate minute amounts of protein. For the isolation of membrane proteins cells must be solubilized into detergent micelles. Nonionic salts are preferred, since other agents such as bile salts, precipitate at acid pH or in the presence of bivalent cations.

In an alternative embodiment the antibodies of the present invention are useful for the close juxtaposition of two antigens. This is particularly useful for increasing the localized concentration of antigens, *e.g.*, enzyme-substrate pairs.

4.4 Western Blots

The compositions of the present invention will find great use in immunoblot or western blot analysis. The anti-HEC antibodies may be used as high-affinity primary reagents for the identification of proteins immobilized onto a solid support matrix, such as nitrocellulose, nylon or combinations thereof. In conjunction with immunoprecipitation, followed by gel electrophoresis, these may be used as a single step reagent for use in detecting antigens against which secondary reagents used in the detection of the antigen cause an adverse background. This is especially useful when the antigens studied are immunoglobulins (precluding the use of immunoglobulins binding bacterial cell wall components), the antigens studied cross-react with the detecting agent, or they migrate at the same relative molecular weight as a cross-reacting signal.

Immunologically-based detection methods for use in conjunction with Western blotting include enzymatically-, radiolabel-, or fluorescently-tagged secondary antibodies against the toxin moiety are considered to be of particular use in this regard.

4.5 Vaccines

The present invention contemplates vaccines for use in both active and passive immunization embodiments. Immunogenic compositions, proposed to be suitable for use as a vaccine, may be prepared most readily directly from immunogenic HEC peptides prepared in a manner disclosed herein. Preferably the antigenic material is extensively dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle.

The preparation of vaccines which contain HEC peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference. Typically, such vaccines are prepared as injectables. Either as liquid solutions or suspensions: solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient.

Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines.

Vaccines may be conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides: such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10%, preferably about 1 to about 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 10 to about 95% of active ingredient, preferably about 25 to about 70%.

The HEC-derived peptides of the present invention may be formulated into the vaccine as neutral or salt forms. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the peptide) and those which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, *e.g.*, the capacity of the individual's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination. Suitable regimes for initial administration and booster shots are also variable, but

are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the size of the host.

Various methods of achieving adjuvant effect for the vaccine includes use of agents such as aluminum hydroxide or phosphate (alum), commonly used as about 0.05 to about 0.1% solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol®) used as an about 0.25% solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between about 70° to about 101°C for a 30-second to 2-minute period, respectively. Aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of Gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with a 20% solution of a perfluorocarbon (Fluosol-DA®) used as a block substitute may also be employed.

In many instances, it will be desirable to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be at from two to twelve week intervals, more usually from three to five week intervals. Periodic boosters at intervals of 1-5 years, usually three years, will be desirable to maintain protective levels of the antibodies. The course of the immunization may be followed by assays for antibodies for the supernatant antigens. The assays may be performed by labeling with conventional labels, such as radionuclides, enzymes, fluorescents, and the like. These techniques are well known and may be found in a wide variety of patents, such as U.S. Patent Nos. 3,791,932; 4,174,384 and 3,949,064, as illustrative of these types of assays.

4.6 DNA Segments

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous,

promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with a DNA segment encoding a HEC peptide in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any viral, prokaryotic (*e.g.*, bacterial), eukaryotic (*e.g.*, fungal, yeast, plant, or animal) cell, and particularly those of mammalian cells. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.*, 1989. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. Appropriate promoter/expression systems contemplated for use in high-level expression include, but are not limited to, the *Pichia* expression vector system (Pharmacia LKB Biotechnology), a baculovirus system for expression in insect cells, or any suitable yeast or bacterial expression system.

In connection with expression embodiments to prepare recombinant proteins and peptides, it is contemplated that longer DNA segments will most often be used, with DNA segments encoding the entire peptide sequence being most preferred. However, it will be appreciated that the use of shorter DNA segments to direct the expression of HEC peptides or epitopic core regions, such as may be used to generate anti-HEC antibodies, also falls within the scope of the invention. DNA segments that encode HEC peptide antigens from about 10 to about 100 amino acids in length, or more preferably, from about 20 to about 80 amino acids in length, or even more preferably, from about 30 to about 70 amino acids in length are contemplated to be particularly useful.

In addition to their use in directing the expression of HEC peptides of the present invention, the nucleic acid sequences contemplated herein also have a variety of other uses. For example, they also have utility as probes or primers in nucleic acid hybridization embodiments. As such, it is contemplated that nucleic acid segments that comprise a sequence region that consists of at least an about 14-nucleotide long contiguous sequence that has the same sequence as, or is complementary to, an about 14-nucleotide long contiguous DNA segment of SEQ ID NO:2 will find particular utility. Longer contiguous identical or complementary sequences, *e.g.*,

those of about 20, 30, 40, 50, 100, 200, (including all intermediate lengths) and even those up to and including about 220-bp (full-length) sequences will also be of use in certain embodiments.

The ability of such nucleic acid probes to specifically hybridize to HEC-encoding sequences will enable them to be of use in detecting the presence of complementary sequences in a given sample. However, other uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

Nucleic acid molecules having sequence regions consisting of contiguous nucleotide stretches of about 14, 15-20, 30, 40, 50, or even of about 100 to about 200 nucleotides or so, identical or complementary to the DNA sequence of SEQ ID NO:1, are particularly contemplated as hybridization probes for use in, *e.g.*, Southern and Northern blotting. Smaller fragments will generally find use in hybridization embodiments, wherein the length of the contiguous complementary region may be varied, such as between about 10-14 and up to about 100 nucleotides, but larger contiguous complementarity stretches may be used, according to the length complementary sequences one wishes to detect.

The use of a hybridization probe of about 14 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Molecules having contiguous complementary sequences over stretches greater than 14 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of about 15 to about 20 contiguous nucleotides, or even longer where desired.

Of course, fragments may also be obtained by other techniques such as, *e.g.*, by mechanical shearing or by restriction enzyme digestion. Small nucleic acid segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced using an automated oligonucleotide synthesizer. Also, fragments may be obtained by application of nucleic acid reproduction technology, such as PCR, by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA techniques generally known to those of skill in the art of molecular biology.

Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of DNA fragments. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, *e.g.*, conditions of high stringency where one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50°C to about 70°C. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating HEC-encoding DNA segments. Detection of DNA segments *via* hybridization is well-known to those of skill in the art, and the teachings of U.S. Patents 4,965,188 and 5,176,995 (each incorporated herein by reference) are exemplary of the methods of hybridization analyses. Teachings such as those found in the texts of Maloy *et al.*, 1994; Segal, 1976; Prokop, 1991; and Kuby, 1994, are particularly relevant.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate HEC-encoding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ conditions such as about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one will likely desire to employ a

fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantitated, by means of the label.

4.7 Biological Functional Equivalents

Modification and changes may be made in the structure of the peptides of the present invention and DNA segments which encode them and still obtain a functional molecule that encodes a protein or peptide with desirable characteristics. The following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. The amino acid changes may be achieved by changing the codons of the DNA sequence, according to the following codon table:

TABLE 1

Amino Acids			Codons			
Alanine	Ala	A	GCA	GCC	GCG	GCU
Cysteine	Cys	C	UGC	UGU		
Aspartic acid	Asp	D	GAC	GAU		
Glutamic acid	Glu	E	GAA	GAG		
Phenylalanine	Phe	F	UUC	UUU		
Glycine	Gly	G	GGA	GGC	GGG	GGU

TABLE 1 - Continued

Amino Acids		Codons						
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein

with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include:

arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

4.8 Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double stranded vector which includes within its sequence a DNA sequence which encodes the desired peptide. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector

is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

4.9 Monoclonal Antibodies

Means for preparing and characterizing antibodies are well known in the art (See, *e.g.*, Harlow and Lane, 1988; incorporated herein by reference).

The methods for generating monoclonal antibodies (mAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic composition in accordance with the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, *m*-maleimidobenzoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a

non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster, injection may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate mAbs.

mAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, *e.g.*, a purified or partially purified LCRF protein, polypeptide or peptide. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B-lymphocytes (B-cells), are selected for use in the mAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was

immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, 1986; Campbell, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 ratio, though the ratio may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described (Kohler and Milstein, 1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.*, (1977). The use of electrically induced fusion methods is also appropriate (Goding, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about 1×10^{-6} to 1×10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B-cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B-cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide mAbs. The cell lines may be exploited for mAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide mAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the mAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. mAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

4.10 Pharmaceutical Compositions

The pharmaceutical compositions disclosed herein may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal

tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of the unit. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

The active compounds may also be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable

mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

The composition can be formulated in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such

organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

5.0 EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes of practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.1 Example 1--Isolation of Full-length cDNA

A 1.8 kb C15 cDNA fragment was originally cloned from a human B cell cDNA library by its interaction with the C-terminus of Rb in a yeast two-hybrid system (Durfee *et al.*, 1993). This fragment was then used as a probe to screen a cDNA library and to obtain several overlapping cDNA clones. The longest clone thus obtained, spanning ~2.3 kb, was ligated into pBKS to create pBKS-C15, from which the *HEC* cDNA was sequenced and from which the longest open reading frame was deduced.

A single 2.3 kb mRNA species was found in all cells tested, using the C15 fragment as a probe (FIG. 1A). Most of the tumor cell lines expressed C15 mRNA more abundantly compared with normal tissues and untransformed cells (FIG. 1B). This expression pattern led to the name HEC (highly expressed in cancer). To test whether the expression in cells changed with progression of the cell cycle, RNA was prepared from synchronized CV1 monkey kidney cells at different time points after G1 arrest. Total RNA extraction, polyA mRNA isolation, and RNA blotting analysis were performed according to standard methods (Chen *et al.*, 1989). The Gb-like and E2F-1 mRNA probes have been described previously (Shan *et al.*, 1992). Gb-like mRNA is expressed constitutively and served as an internal loading control (Gullemont *et al.*, 1989). Monkey kidney CV1 cells treated with drugs to enrich for distinct cell cycle stages were used for RNA extraction as described (Shan *et al.*, 1992).

HEC mRNA expression varied with the cell cycle, increasing during S and M phases (FIG. 1C); such an expression pattern is somewhat different from that of the transcription factor E2F-1, for which expression peaks at G1/S and decreases in M (Shan *et al.*, 1992). These results suggested that the protein encoded by *HEC* may normally play a role in cell proliferation.

To obtain the full-length *HEC* cDNA, the original 1.8 kb C15 cDNA fragment was used as a probe to screen a human B cell cDNA library. Several clones containing different fragments about 2 kb in length were sequenced. The longest clone revealed an open reading frame encoding a 642-amino acid protein with a predicted molecular weight of 72 kD (FIG. 2A). The protein was acidic, with an isoelectric point of 5.5.

A search of the updated GenBank revealed no significant homology with any gene encoding a characterized protein.

5.2 Example 2--Isolation and Identification of HEC protein

A GST-C15 fusion protein containing amino acids 56-642 was created by ligating a unique *Xho*I-*Xho*I *C15* cDNA fragment (nucleotides 264-2045) into a modified version of pGEX-3X (Stratagene, San Diego, CA) to create pGST-C15. The protein was expressed in *E. coli*, induced by isopropyl-b-D-thiogalactopyranoside (0.1 mM), and purified with glutathione-sepharose beads as described (Chen, P.-L. *et al.*, 1995). Recovered protein, >95% pure, was then used as an antigen in mice. Serum from the immunized mice was preabsorbed on GST columns and used directly for immunoprecipitation, developing immunoblots, and immunostaining. Preimmune serum was obtained from the same mice and used at the same dilution (1:1000). Monoclonal antibodies were prepared according to standard procedures (Harlow and Lane, 1988) and characterized as above.

To identify the cellular protein encoded by *HEC*, polyclonal antibodies raised against a GST-C15 fusion protein were prepared. T24 bladder carcinoma cells were metabolically labeled with ^{35}S-methionine and the cell lysates were then prepared for immunoprecipitation. To label cellular proteins, T24 cells (5×10^6 for each lane) were grown to ~70% confluence, then incubated with ^{35}S-methionine (300 mCi) for 2 hours. Cells were then lysed in Lysis 250 buffer (Chen, Y. *et al.*, 1996) for immunoprecipitation. For *in vitro* translation, the full-length *HEC* cDNA was inserted into pBKS, then transcribed and translated in the presence of ^{35}S-methionine, using the TNT coupled reticulocyte lysate system (Promega, Madison, WI).

Anti-C15 antibodies specifically immunoprecipitated a cellular protein that migrated in SDS-PAGE with an apparent molecular mass of 76 kD (FIG. 2B, lane 5). The protein was not detected by preimmune serum (lane 4). Furthermore, a GST-C15 fusion protein competed in the immunopreciptation for binding to the antibodies and completely prevented precipitation of the cellular protein (lane 6). An unknown 46 kD protein was co-immunoprecipitated. This could be one of the HEC interacting proteins as described below (FIG. 2B, lane 5).

When full-length *HEC* cDNA was used as template for *in vitro* transcription and translation, the synthesized protein was also immunoprecipitated by the same antibodies, and migrated at the same position as the cellular protein (FIG. 2B, lane 3). These results showed cellular HEC to be a 76 kD protein, and are consistent with the size predicted closely from the full-length *HEC* cDNA. A striking feature of the protein is its long series of typical leucine

heptad repeats. These repeats span the region between amino acids 254 and 621, nearly two-thirds of the entire protein.

5.3 Example 3--Expression of HEC Protein in Actively Dividing Cells

To evaluate the expression pattern of HEC, protein lysates prepared from different organs of an adult mouse were used for straight Western blotting analysis (FIG. 3A). HEC protein could be detected only in tissues with high mitotic indices, such as testis, spleen, and thymus (FIG. 3A, upper panel). The internal control protein, p84 (Durfee *et al.*, 1994), was expressed in approximately equivalent amounts in all of these tissues. The expression of HEC in tissues with high mitotic indices is consistent with the mRNA expression pattern, and suggested a potential role for HEC in proliferation in general or mitosis in particular.

To confirm this notion further, the expression pattern of HEC was monitored during cell cycle progression. Human bladder carcinoma cells, T24, grown in DMEM/10% FCS, were synchronized at G1 by density arrest in DMEM/0.5% serum, then released at time zero by replating in DMEM/10% FCS at a density of 2×10^6 cells per 10 cm plate. At various time points thereafter (18 hr for G1/S, 22 hr for S, 33 hr for G2), cells were harvested. To obtain cells in M phase, nocodazole (0.4 mg/ml) was added to culture medium for 8 hours prior to harvest. Samples of cells were fixed in ethanol and analysed using fluorescence activated cell sorting to determine cell cycle phases as described (Chen, Y. *et al.*, 1996).

Cell lysates prepared from a synchronized population of T24 cells were analyzed by Western blotting with three different antibodies, mAb 11D7 (Rb) (Shan *et al.*, 1992), anti-C15 (HEC), and anti-N5 (p84) (Durfee *et al.*, 1994). HEC protein is expressed in detectable amounts in late S to M (FIG. 3B). The Rb expression pattern in different cell cycle phases has previously been described (Chen *et al.*, 1989), and served as a marker for cell cycle phases. p84 expression does not vary with progression of the cell cycle and served as an internal loading control (Durfee *et al.*, 1994). In rapidly dividing U937 monocytic lymphoma cells HEC was expressed and detectable.

Mouse fibroblasts 3T3/L1 were induced to differentiate as described previously by Student *et al.* (1980). This induction involved first growing cells to confluence, then, at day 0 of the differentiation induction program, exposing them to fresh DMEM containing 10% FBS, 1 mM dexamethasone, 10 mM forskolin, and 10 mg/ml insulin for 48 hr to initiate

adipogenesis. The medium was then replaced with DMEM containing 10% FBS and 10 mg/ml insulin, and cells were refed every other day until day 8. To confirm the appearance of the adipogenic phenotype, particularly the accumulation of fat droplets in the cytoplasm, cells were fixed at particular time points in 3% glutaraldehyde/100 mM sodium phosphate (pH 7.4) and stained with Oil Red EGN.

Similarly, U937 cells at an initial density of 5×10^5 per ml were incubated for 4 days in the presence of phorbol ester (TPA, 100 ng/ml). Macrophages were observed at day four as previously described (Chen *et al.*, 1989). When these cells were induced by phorbol ester to differentiate terminally into macrophages (Chen, P.-L. *et al.*, 1996; Sundstrum and Nilsson, 1976), however (FIG. 3C, lane 4), HEC expression fell to undetectable amounts. Likewise, when murine 3T3/L1 cells were induced by appropriate hormones to differentiate into adipocytes (Chen, *et al.*, 1989; Student *et al.*, 1980) (FIG. 3D), HEC expression was easily detectable in dividing cells (FIG. 3D, lanes U, 1) but or undetectable in cells arrested at G0/G1 (lane 0) or differentiated terminally (lanes 4-6). These results, showing that HEC was not expressed in terminally differentiated cells, further strengthened the suggestion that HEC may function specifically in mitosis.

5.4 Example 4--HEC Localization

In order to assess the potential function of HEC in dividing cells, the subcellular location of HEC was determined. T24 cells were fractionated biochemically or fixed and immunostained with specific anti-HEC antibodies. The procedures to separate membrane, nuclear, and cytoplasmic fractions were adapted from those previously published (Abrams *et al.*, 1982). All three fractions were then assayed for Rb protein and HEC by immunoprecipitation as described above. Aliquots of each fraction were also incubated with glutathione-agarose beads, then separated with SDS-PAGE and stained with Coomassie blue. Glutathione-S-transferase was thus identified as a cytoplasmic marker (Gullemont *et al.*, 1989).

In cells biochemically fractionated into nuclei, cytoplasm, and membrane components (Abrams *et al.*, 1982), HEC distributes mainly with the nuclear fraction (FIG. 4A). Rb, a nuclear protein, and glutathione transferase, a cytoplasmic protein, served to control the fractionation procedure. By immunocytochemical staining methods, HEC also localizes in the nucleus in a speckled pattern (FIG. 4B, a, b, and c). In mitotic cells, the protein localized as

paired dots on chromosomes (FIG. 4B, panel d and FIG 4C, panel a). Staining of metaphase chromosome spreads revealed co-localization with centromere proteins (CENPs) recognized by serum from a patient with autoimmune disease and CREST syndrome (Moroi *et al.*, 1980) (FIG. 4C). The use of preimmune serum resulted in the lack of any fluorescent signal, and demonstrated that the anti-HEC antibodies are indeed specific. These results show HEC to be a nuclear, centromere-associated protein, and again suggest a role for HEC in M phase.

5.5 Example 5--Effect of truncated HEC on Tubulin Formation

The long stretches of leucine heptad repeats of HEC suggested that the C-terminal portion of the protein might be crucial for binding other proteins (Landschulz *et al.*, 1988). The inventors reasoned that ectopic expression of a HEC mutant containing only the heptad repeats might bind or compete with endogenous HEC for binding to other proteins, and thereby influence mitosis.

To test this hypothesis, two HEC mutants fused to green-florescence protein (GFP) and under the regulation of the CMV immediate early gene promoter were constructed. One, GFP-15PA, contained the N-terminal region only (amino acids 1 to 250); the other, GFP-15Pst, contained the entire series of leucine heptad repeats (a.a. 251 to 618) (FIG. 5A). The GFP plasmid construct alone served as a control. Transfection of these three constructs into Rb-negative Saos-2 cells resulted in expression of the corresponding proteins, which could be detected by first immunoprecipitation with anti-myc tag antibody and then Western blotting using anti-GFP antibody as probe (FIG. 5B, lanes 2-4).

Twenty four hours after transfection, cells were observed directly using fluorescence microscopy. Expression of GFP was observed in nuclei and cytoplasm (FIG. 5C, panel *b*), while GFP-15PA was observed only in nuclei (FIG. 5C, panel *e*), and GFP-15Pst only in cytoplasm (FIG. 5C, panel *h*).

Cells grown on coverslips in tissue culture dishes were washed in phosphate buffered saline (PBS) and fixed for 30 minutes in 4% formaldehyde in PBS with 0.5% Triton X-100. After treating with 0.05% Saponin in water for 30 minutes and extensively washing with PBS, cells were blocked in PBS containing 10% normal goat serum. A one hour incubation with suitable antibody diluted in 10% goat serum was followed by three washes, then by another one-hour incubation with fluorochrome-conjugated secondary antibody. Co-localization of

HEC and CENPs was performed using a polyclonal mouse anti-C15 antibody mixed with human CREST autoimmune sera. Polyclonal (ICN, Costa Mesa, CA) rabbit or DM 1A monoclonal (Sigma, St. Louis, MO) anti-tubulin antibodies were used to localize tubulin. The respective antigens were visualized with goat anti-human IgG or goat-anti-rabbit IgG conjugated to Texas Red and goat anti-mouse conjugated to FITC. After washing extensively in PBS with 0.5% Nonidet-P 40, cells were further stained with the DNA specific dye 4',6-diamidino-2 phenylindole (DAPI) and mounted in Permafluor (Lipshaw-Immunonon, Inc., Pittsburgh, PA). Ektachrome P1600 film was used when pictures were taken from a standard fluorescence microscope (Axiophot Photomicroscope, Zeiss).

When the cells were immunostained with an anti-tubulin mAb, tubulin localized almost exclusively in the nuclei of cells expressing GFP-15Pst (FIG. 5C, panel *i*). In contrast, tubulin was found predominantly in cytoplasm of cells transfected with both GFP and GFP-15PA(FIG. 5C, panels *c* and *f*). Normally, spindle-associated tubulin should be completely degraded after mitosis, and the tubulin present in interphase cells should be distributed only in the cytoplasm. However, tubulin localized abnormally within the nuclei of cells expressing the GFP-15Pst HEC mutant protein, (FIG. 5C, panel *i*), indicating that the ectopic expression of N-terminally-truncated HEC mutant disturbed the machinery that degrades or re-distributes tubulin after mitosis.

To further study the effects of the HEC mutants, individual cells expressing GFP and GFP-HEC mutant fusions were followed serially and scored for their abilities to divide during the 99 hour period after transfection. Three constructs, CNPL-GFP, which was a derivative plasmid from a mammalian expressing vector containing myc-tagged mutant form of green fluorescence protein (S65T) (Heim *et al.*, 1994); CNPL-GFP-15PA, containing GFP fused to the N-terminus of HEC (a.a. 1-250); and CNPL-GFP-15Pst, containing GFP fused to C-terminal HEC (a.a. 251-618), were used in the transient transfection assays. Transfections were carried out on 1×10^6 cells at a time by conventional calcium phosphate/DNA co-immunoprecipitation. The precipitates were removed 12 hours after transfection and the cultures were refed with fresh medium. The cells were observed under a fluorescence microscope.

Substantial numbers of Saos-2 cells expressing either GFP or GFP-15PA were able to divide into four-cell colonies (FIG. 6A, B). Expression of GFP-15Pst in Saos-2 cells, however,

not only abolished the appearance of four-cell colonies but also reduced the number of two-cell colonies substantially (FIG. 6C).

Human bladder carcinoma T24 and monkey kidney CV1 cell were also used in identical studies, and similar results were obtained. It was noted that many of the cells expressing the GFP-15Pst HEC mutant passed through the first mitosis, but were largely unable to complete the next round of cell division. This observation is consistent with the abnormal distribution of tubulin in the same cells; tubulin in the nuclei indicates an abnormality in orchestration of the previous mitosis.

5.6 Example 6--Effect of Anti-HEC Antibodies on M-Phase

To test directly whether HEC is important functionally for M phase, an alternative approach was employed to inactivate HEC. First, mouse monoclonal antibody 9G3 was generated using the same GST-C15 fusion protein immunogen used to make the polyclonal antiserum. This mAb was specific for HEC in immunoprecipitation and straight immunoblotting (FIG. 7A); it recognizes the same 76 kD protein as the polyclonal anti-C15 serum.

T24 human bladder carcinoma cells synchronized in S phase were microinjected with mAb 9G3 monoclonal antibodies. Cells were injected with antibody solutions at concentrations of 2 mg/ml in microinjection buffer [20 mM NaHPO4 (pH 7.2), 0.1 mM EDTA, 10% glycerol], using Eppendorf's microinjection apparatus as described (Goodrich *et al.*, 1991).

Twenty-six hours later, at a time when all cells should have completed mitosis, the majority of cells injected with mAb 9G3 contained multiple, fragmented nuclei. Uninjected cells and those injected with the control antibody (total murine IgG) divided into two normal daughter cells (FIG. 7B). Many cells injected with anti-HEC antibodies were missing and presumed dead 26 hours after injection. Results of three separate studies were consistent (Table†2).

TABLE 2

Summary of results from microinjection of T24 cells with anti-HEC monoclonal antibody mAb 9G3

Experiment No.	Injected antibody	Divided cells		Undivided cells	Total
		abnormal	normal		
1	mouse IgG	0	122	22	144
	mAb 9G3	108	0	27	135
2	mouse IgG	0	76	16	92
	mAb 9G3	58	0	16	74
3	mouse IgG	0	171	28	199
	mAb 9G3	92	0	13	105
Total	mouse IgG	0	369	66	435
	mAb 9G3	258	0	56	314

M phase progression in cells injected with anti-HEC monoclonal antibodies was observed closely to determine more precisely the events responsible for the appearance abnormal nuclei and cell death. Chromosomes in anti-HEC injected cells condensed but failed to congress or segregate properly (FIG. 7C). No distinct metaphase plates were observed, and spindles were disorganized in relation to their centromeres (FIG. 7C, panels *g-l*). In many cells, multiple spindle poles were observed (FIG. 7C, panels *g-h*). Superimposition of images of the same cells stained with DAPI (to identify chromosomes) and with an antibody recognizing tubulin (to identify the spindle apparatus) showed that many spindles failed to assume the proper orthogonal orientation to their chromatids (FIG. 7C, panels *k*, *l*). Cells injected with anti-HEC were able to undergo cytokinesis, but chromosomes separated haphazardly into grossly abnormal daughter cells which were ultimately nonviable.

5.7 Example 7--HEC Interaction With Other Proteins

The effects on mitosis of overexpression of the HEC mutant indicated that the leucine hepatad repeats of HEC were critical to the protein's function. To explore the potential biochemical basis for the abnormal mitosis after HEC inactivation, the inventors searched for proteins with which HEC interacts.

The C-terminal half of HEC (a.a. 251-618), which includes the long stretch of leucine heptad repeats, was employed as a bait to perform yeast two-hybrid screens in a human lymphocyte cDNA library. Among the 16 strongly interacting clones in this screen, 10 were identified as cDNA fragments encoding MSS1, a component of subunit 7 of the 26S proteasome (Dubiel *et al.*, 1993; Shibuya *et al.*, 1992). Others encoded subunit p45 of the 26S proteasome complex (Akiyama *et al.*, 1995); Sb1.8, the human homolog of yeast Smc1/Smc2 (Rocques *et al.*, 1995, Strunnikov *et al.*, 1993 and 1995); and Nek2 (Schultz and Nigg 1993), the human homolog of NimA in *Aspergillus nidulans* (Osmani *et al.*, 1988), a kinase crucial for the progression of M phase (Table 3).

TABLE 3

Summary of cellular proteins interacting with HEC

HEC-Aps clones	Binding in yeast[a]	*In vitro* binding[b]	*In vivo* binding[c]	Identity	Homology	Mutant phenotype
1,6,7,8...	371.5 ± 19.3	+	+	mss1	cim5	G2/M arrest
2	468.9 ± 65.8	ND	ND	Novel		
4	273.3 ± 10.0	+	ND	sb1.8	smc1/smc2	G2/M arrest
5	620.4 ± 165.8	+	ND	Novel		
14	239.4 ± 32.6	+	+	nek2	nimA	G2/M arrest
20	319.1 ± 16.6	+	ND	Novel		
24	1105.2 ± 159.3	+	ND	Subunit p45 of 26S proteasome	cim3(sug1)	G2/M arrest

a; β-galatosidase activity, arbitrary units compared to the control host yeast Y153 alone.

b; *In vitro* binding was determined by GST pull-down assay with *in vitro* translated, full-length HEC.

c; *In vivo* binding was determined by reciprocal co-immunoprecipitation.

ND: Not done.

All four of these proteins also interacted with HEC by either co-immunoprecipitation or GST pull-down assays (Table 3). All of the characterized, HEC-associated proteins have been linked genetically to M phase. These interacting proteins provided additional evidence to implicate HEC in the regulation of events important for faithful proportioning of chromosomes to daughter cells during M phase.

5.8 Example 8--Materials and Methods

Immunoprecipitation and Western Blot Analysis

Cell lysates in Lysis 250 buffer were subjected to three freeze/thaw cycles (liquid nitrogen/37°C), and clarified by centrifugation (14,000 rpm, 2 min at room temperature). The supernatants were used for immunoprecipitation as described (Chen, P.-L. *et al.*, 1996). Briefly, to each clarified supernatant was added 1 ml of mouse polyclonal anti-C15 antisera. For competition studies, antigens and antibodies were incubated together for 1 hr before addition to the cell lysate. After 1-hr incubation, protein-A sepharose beads were added for another hour. Beads were then collected and washed 5 times with lysis buffer containing 250 mM NaCl and then boiled in SDS-loading buffer for immunoblotting analysis as described (Chen, Y. *et al.*, 1996).

Metaphase Chromosome Spreads

Growing T24 cells were treated with nocodazole for 8 hr. Mitotic cells were shaken off the culture plates and hypotonically lysed in 75 mM KCl. Free chromosomes were then cytospun onto coverslips and incubated with a drop of DAPI for 10 minutes. The same chromosomes were stained with anti-HEC and human autoimmune (CREST) antisera, then counterstained with FITC- or Texas Red-conjugated secondary antibodies. Digital photographs were obtained using a Ziess microscope (magnification 400 ×) and a Hamamatsu Photonics camera. Images were superimposed using Photoshop for Power MacIntosh software. Similar results were obtained when a confocal imaging system was applied.

Materials:

The CREST antiserum was obtained from Dr. B. Brinkley **(COMPANY, CITY, STATE)**. The GFP plasmid was obtained from Dr. R. Tsien**(COMPANY, CITY, STATE**

Yeast two-hybrid screen

A yeast two-hybrid system (Durfee *et al.*, 1993), modified as described as pAS-15Pst, which contains amino acids 251-618 of HEC, was used as bait.

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(ii) TITLE OF INVENTION: PROTEINS AND COMPOSITIONS FOR MODULATING MITOSIS

(iii) NUMBER OF SEQUENCES: 2:

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2090 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:105..2030

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CTCGAGCCAC GAAGGCCCCG CTGTCCTGTC TAGCAGATAC TTGCACGGTT TACAGAAATT      60

CGGTCCCTGG GTCGTGTCAG GAAACTGGAA AAAAGGTCAT AAGC ATG AAG CGC AGT      116
                                                 Met Lys Arg Ser
                                                   1

TCA GTT TCC AGC GGT GGT GCT GGC CGC CTC TCC ATG CAG GAG TTA AGA      164
Ser Val Ser Ser Gly Gly Ala Gly Arg Leu Ser Met Gln Glu Leu Arg
  5                  10                  15                  20

TCC CAG GAT GTA AAT AAA CAA GGC CTC TAT ACC CCT CAA ACC AAA GAG      212
Ser Gln Asp Val Asn Lys Gln Gly Leu Tyr Thr Pro Gln Thr Lys Glu
                 25                  30                  35
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Lys Pro Thr Phe Gly Lys Leu Ser Ile Asn Lys Pro Thr Ser Glu Arg
             40                  45                  50

AAA GTC TCG CTA TTT GGC AAA AGA ACT AGT GGA CAT GGA TCC CGG AAT       308
Lys Val Ser Leu Phe Gly Lys Arg Thr Ser Gly His Gly Ser Arg Asn
         55                  60                  65

AGT CAA CTT GGT ATA TTT TCC AGT TCT GAG AAA ATC AAG GAC CCG AGA       356
Ser Gln Leu Gly Ile Phe Ser Ser Ser Glu Lys Ile Lys Asp Pro Arg
     70                  75                  80

CCA CTT AAT GAC AAA GCA TTC ATT CAG CAG TGT ATT CGA CAA CTC TGT       404
Pro Leu Asn Asp Lys Ala Phe Ile Gln Gln Cys Ile Arg Gln Leu Cys
 85                  90                  95                 100

GAG TTT CTT ACA GAA AAT GGT TAT GCA CAT AAT GTG TCC ATG AAA TCT       452
Glu Phe Leu Thr Glu Asn Gly Tyr Ala His Asn Val Ser Met Lys Ser
                105                 110                 115

CTA CAA GCT CCC TCT GTT AAA GAC TTC CTG AAG ATC TTC ACA TTT CTT       500
Leu Gln Ala Pro Ser Val Lys Asp Phe Leu Lys Ile Phe Thr Phe Leu
            120                 125                 130

TAT GGC TTC CTG TGC CCC TCA TAC GAA CTT CCT GAC ACA AAG TTT GAA       548
Tyr Gly Phe Leu Cys Pro Ser Tyr Glu Leu Pro Asp Thr Lys Phe Glu
        135                 140                 145

GAA GAG GTT CCA AGA ATC TTT AAA GAC CTT GGG TAT CCT TTT GCA CTA       596
Glu Glu Val Pro Arg Ile Phe Lys Asp Leu Gly Tyr Pro Phe Ala Leu
    150                 155                 160

TCC AAA AGC TCC ATG TAC ACA GTG GGG GCT CCT CAT ACA TGG CCT CAC       644
Ser Lys Ser Ser Met Tyr Thr Val Gly Ala Pro His Thr Trp Pro His
165                 170                 175                 180

ATT GTG GCA GCC TTA GTT TGG CTA ATA GAC TGC ATC AAG ATA CAT ACT       692
Ile Val Ala Ala Leu Val Trp Leu Ile Asp Cys Ile Lys Ile His Thr
                185                 190                 195

GCC ATG AAA GAA AGC TCA CCT TTA TTT GAT GAT GGG CAG CCT TGG GGA       740
Ala Met Lys Glu Ser Ser Pro Leu Phe Asp Asp Gly Gln Pro Trp Gly
            200                 205                 210

GAA GAA ACT GAA GAT GGA ATT ATG CAT AAT AAG TTG TTT TTG GAC TAC       788
Glu Glu Thr Glu Asp Gly Ile Met His Asn Lys Leu Phe Leu Asp Tyr
        215                 220                 225

ACC ATA AAA TGC TAT GAG AGT TTT ATG AGT GGT GCC GAC AGC TTT GAT       836
Thr Ile Lys Cys Tyr Glu Ser Phe Met Ser Gly Ala Asp Ser Phe Asp
    230                 235                 240

GAG ATG AAT GCA GAG CTG CAG TCA AAA CTG AAG GAT TTA TTT AAT GTG       884
Glu Met Asn Ala Glu Leu Gln Ser Lys Leu Lys Asp Leu Phe Asn Val
245                 250                 255                 260
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GAT GCT TTT AAG CTG GAA TCA TTA GAA GCA AAA AAC AGA GCA TTG AAT      932
Asp Ala Phe Lys Leu Glu Ser Leu Glu Ala Lys Asn Arg Ala Leu Asn
                265                 270                 275

GAA CAG ATT GCA AGA TTG GAA CAA GAA AGA GAA AAA GAA CCG AAT CGT      980
Glu Gln Ile Ala Arg Leu Glu Gln Glu Arg Glu Lys Glu Pro Asn Arg
            280                 285                 290

CTA GAG TCG TTG AGA AAA CTG AAG GCT TCC TTA CAA GGA GAT GTT CAA     1028
Leu Glu Ser Leu Arg Lys Leu Lys Ala Ser Leu Gln Gly Asp Val Gln
        295                 300                 305

AAG TAT CAG GCA TAC ATG AGC AAT TTG GAG TCT CAT TCA GCC ATT CTT     1076
Lys Tyr Gln Ala Tyr Met Ser Asn Leu Glu Ser His Ser Ala Ile Leu
    310                 315                 320

GAC CAG AAA TTA AAT GGT CTC AAT GAG GAA ATT GCT AGA GTA GAA CTA     1124
Asp Gln Lys Leu Asn Gly Leu Asn Glu Glu Ile Ala Arg Val Glu Leu
325                 330                 335                 340

GAA TGT GAA ACA ATA AAA CAG GAG AAC ACT CGA CTA CAG AAT ATC ATT     1172
Glu Cys Glu Thr Ile Lys Gln Glu Asn Thr Arg Leu Gln Asn Ile Ile
                345                 350                 355

GAC AAC CAG AAG TAC TCA GTT GCA GAC ATT GAG CGA ATA AAT CAT GAA     1220
Asp Asn Gln Lys Tyr Ser Val Ala Asp Ile Glu Arg Ile Asn His Glu
            360                 365                 370

AGA AAT GAA TTG CAG CAG ACT ATT AAT AAA TTA ACC AAG GAC CTG GAA     1268
Arg Asn Glu Leu Gln Gln Thr Ile Asn Lys Leu Thr Lys Asp Leu Glu
        375                 380                 385

GCT GAA CAA CAG AAG TTG TGG AAT GAG GAG TTA AAA TAT GCC AGA GGC     1316
Ala Glu Gln Gln Lys Leu Trp Asn Glu Glu Leu Lys Tyr Ala Arg Gly
    390                 395                 400

AAA GAA GCG ATT GAA ACA CAA TTA GCA GAG TAT CAC AAA TTG GCT AGA     1364
Lys Glu Ala Ile Glu Thr Gln Leu Ala Glu Tyr His Lys Leu Ala Arg
405                 410                 415                 420

AAA TTA AAA CTT ATT CCT AAA GGT GCT GAG AAT TCC AAA GGT TAT GAC     1412
Lys Leu Lys Leu Ile Pro Lys Gly Ala Glu Asn Ser Lys Gly Tyr Asp
                425                 430                 435

TTT GAA ATT AAG TTT AAT CCC GAG GCT GGT GCC AAC TGC CTT GTC AAA     1460
Phe Glu Ile Lys Phe Asn Pro Glu Ala Gly Ala Asn Cys Leu Val Lys
            440                 445                 450

TAC AGG GCT CAA GTT TAT GTA CCT CTT AAG GAA CTC CTG AAT GAA ACT     1508
Tyr Arg Ala Gln Val Tyr Val Pro Leu Lys Glu Leu Leu Asn Glu Thr
        455                 460                 465

GAA GAA GAA ATT AAT AAA GCC CTA AAT AAA AAA ATG GGT TTG GAG GAT     1556
Glu Glu Glu Ile Asn Lys Ala Leu Asn Lys Lys Met Gly Leu Glu Asp
    470                 475                 480
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ACT TTA GAA CAA TTG AAT GCA ATG ATA ACA GAA AGC AAG AGA AGT GTG      1604
Thr Leu Glu Gln Leu Asn Ala Met Ile Thr Glu Ser Lys Arg Ser Val
485                 490                 495                 500

AGA ACT CTG AAA GAA GAA GTT CAA AAG CTG GAT GAT CTT TAC CAA CAA      1652
Arg Thr Leu Lys Glu Glu Val Gln Lys Leu Asp Asp Leu Tyr Gln Gln
                505                 510                 515

AAA ATT AAG GAA GCA GAG GAA GAG GAT GAA AAA TGT GCC AGT GAG CTT      1700
Lys Ile Lys Glu Ala Glu Glu Glu Asp Glu Lys Cys Ala Ser Glu Leu
            520                 525                 530

GAG TCC TTG GAG AAA CAC AAG CAC CTG CTA GAA AGT ACT GTT AAC CAG      1748
Glu Ser Leu Glu Lys His Lys His Leu Leu Glu Ser Thr Val Asn Gln
        535                 540                 545

GGG CTC AGT GAA GCT ATG AAT GAA TTA GAT GCT GTT CAG CGG GAA TAC      1796
Gly Leu Ser Glu Ala Met Asn Glu Leu Asp Ala Val Gln Arg Glu Tyr
    550                 555                 560

CAA CTA GTT GTG CAA ACC ACG ACT GAA GAA AGA CGA AAA GTG GGA AAT      1844
Gln Leu Val Val Gln Thr Thr Thr Glu Glu Arg Arg Lys Val Gly Asn
565                 570                 575                 580

AAC TTG CAA CGT CTG TTA GAG ATG GTT GCT ACA CAT GTT GGG TCT GTA      1892
Asn Leu Gln Arg Leu Leu Glu Met Val Ala Thr His Val Gly Ser Val
                585                 590                 595

GAG AAA CAT CTT GAG GAG CAG ATT GCT AAA GTT GAT AGA GAA TAT GAA      1940
Glu Lys His Leu Glu Glu Gln Ile Ala Lys Val Asp Arg Glu Tyr Glu
            600                 605                 610

GAA TGC ATG TCA GAA GAT CTC TCG GAA AAT ATT AAA GAG ATT AGA GAT      1988
Glu Cys Met Ser Glu Asp Leu Ser Glu Asn Ile Lys Glu Ile Arg Asp
        615                 620                 625

AAG TAT GAG AAG AAA GCT ACT CTA ATT AAG TCT TCT GAA GAA              2030
Lys Tyr Glu Lys Lys Ala Thr Leu Ile Lys Ser Ser Glu Glu
    630                 635                 640

TGAAGATAAA ATGTTGATCA TGTATATATA TCCATAGTGA ATAAAATTGT CTCAGTAAAA    2090
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 642 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Lys Arg Ser Ser Val Ser Ser Gly Gly Ala Gly Arg Leu Ser Met
  1               5                  10                  15
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Gln Glu Leu Arg Ser Gln Asp Val Asn Lys Gln Gly Leu Tyr Thr Pro
            20                  25                  30

Gln Thr Lys Glu Lys Pro Thr Phe Gly Lys Leu Ser Ile Asn Lys Pro
        35                  40                  45

Thr Ser Glu Arg Lys Val Ser Leu Phe Gly Lys Arg Thr Ser Gly His
    50                  55                  60

Gly Ser Arg Asn Ser Gln Leu Gly Ile Phe Ser Ser Ser Glu Lys Ile
65                  70                  75                  80

Lys Asp Pro Arg Pro Leu Asn Asp Lys Ala Phe Ile Gln Gln Cys Ile
                85                  90                  95

Arg Gln Leu Cys Glu Phe Leu Thr Glu Asn Gly Tyr Ala His Asn Val
            100                 105                 110

Ser Met Lys Ser Leu Gln Ala Pro Ser Val Lys Asp Phe Leu Lys Ile
        115                 120                 125

Phe Thr Phe Leu Tyr Gly Phe Leu Cys Pro Ser Tyr Glu Leu Pro Asp
    130                 135                 140

Thr Lys Phe Glu Glu Glu Val Pro Arg Ile Phe Lys Asp Leu Gly Tyr
145                 150                 155                 160

Pro Phe Ala Leu Ser Lys Ser Ser Met Tyr Thr Val Gly Ala Pro His
                165                 170                 175

Thr Trp Pro His Ile Val Ala Ala Leu Val Trp Leu Ile Asp Cys Ile
            180                 185                 190

Lys Ile His Thr Ala Met Lys Glu Ser Ser Pro Leu Phe Asp Asp Gly
        195                 200                 205

Gln Pro Trp Gly Glu Glu Thr Glu Asp Gly Ile Met His Asn Lys Leu
    210                 215                 220

Phe Leu Asp Tyr Thr Ile Lys Cys Tyr Glu Ser Phe Met Ser Gly Ala
225                 230                 235                 240

Asp Ser Phe Asp Glu Met Asn Ala Glu Leu Gln Ser Lys Leu Lys Asp
                245                 250                 255

Leu Phe Asn Val Asp Ala Phe Lys Leu Glu Ser Leu Glu Ala Lys Asn
            260                 265                 270

Arg Ala Leu Asn Glu Gln Ile Ala Arg Leu Glu Gln Glu Arg Glu Lys
        275                 280                 285

Glu Pro Asn Arg Leu Glu Ser Leu Arg Lys Leu Lys Ala Ser Leu Gln
    290                 295                 300

Gly Asp Val Gln Lys Tyr Gln Ala Tyr Met Ser Asn Leu Glu Ser His
305                 310                 315                 320
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Ser Ala Ile Leu Asp Gln Lys Leu Asn Gly Leu Asn Glu Glu Ile Ala
                325                 330                 335

Arg Val Glu Leu Glu Cys Glu Thr Ile Lys Gln Glu Asn Thr Arg Leu
            340                 345                 350

Gln Asn Ile Ile Asp Asn Gln Lys Tyr Ser Val Ala Asp Ile Glu Arg
        355                 360                 365

Ile Asn His Glu Arg Asn Glu Leu Gln Gln Thr Ile Asn Lys Leu Thr
    370                 375                 380

Lys Asp Leu Glu Ala Glu Gln Gln Lys Leu Trp Asn Glu Glu Leu Lys
385                 390                 395                 400

Tyr Ala Arg Gly Lys Glu Ala Ile Glu Thr Gln Leu Ala Glu Tyr His
                405                 410                 415

Lys Leu Ala Arg Lys Leu Lys Leu Ile Pro Lys Gly Ala Glu Asn Ser
            420                 425                 430

Lys Gly Tyr Asp Phe Glu Ile Lys Phe Asn Pro Glu Ala Gly Ala Asn
        435                 440                 445

Cys Leu Val Lys Tyr Arg Ala Gln Val Tyr Val Pro Leu Lys Glu Leu
    450                 455                 460

Leu Asn Glu Thr Glu Glu Glu Ile Asn Lys Ala Leu Asn Lys Lys Met
465                 470                 475                 480

Gly Leu Glu Asp Thr Leu Glu Gln Leu Asn Ala Met Ile Thr Glu Ser
                485                 490                 495

Lys Arg Ser Val Arg Thr Leu Lys Glu Glu Val Gln Lys Leu Asp Asp
            500                 505                 510

Leu Tyr Gln Gln Lys Ile Lys Glu Ala Glu Glu Glu Asp Glu Lys Cys
        515                 520                 525

Ala Ser Glu Leu Glu Ser Leu Glu Lys His Lys His Leu Leu Glu Ser
    530                 535                 540

Thr Val Asn Gln Gly Leu Ser Glu Ala Met Asn Glu Leu Asp Ala Val
545                 550                 555                 560

Gln Arg Glu Tyr Gln Leu Val Val Gln Thr Thr Thr Glu Glu Arg Arg
                565                 570                 575

Lys Val Gly Asn Asn Leu Gln Arg Leu Leu Glu Met Val Ala Thr His
            580                 585                 590

Val Gly Ser Val Glu Lys His Leu Glu Glu Gln Ile Ala Lys Val Asp
        595                 600                 605

Arg Glu Tyr Glu Glu Cys Met Ser Glu Asp Leu Ser Glu Asn Ile Lys
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610 615 620

Glu Ile Arg Asp Lys Tyr Glu Lys Lys Ala Thr Leu Ile Lys Ser Ser
625 630 635 640

Glu Glu

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions, methods and steps or in the sequence of steps of the methods described herein without departing from the spirit, scope and concept of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims. Accordingly, the exclusive rights sought to be patented are described in the claims below.

CLAIMS

1. An isolated nucleic acid segment encoding a human nuclear protein (HEC) comprising the amino acid sequence of SEQ ID NO:1.

2. The nucleic acid segment of claim 1 further defined as comprising the nucleic acid sequence of SEQ ID NO:2, or the complement thereof, or a sequence which hybridizes to the sequence of SEQ ID NO:2 under conditions of high stringency.

3. The nucleic acid segment of claim 1 further defined as an RNA segment.

4. An isolated DNA segment comprising a human nuclear protein gene (HEC) that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:1.

5. A recombinant host cell comprising the DNA segment of claim 4.

6. The recombinant cell of claim 5 further defined as a bacterial or eukaryotic cell.

7. The recombinant cell of claim wherein the eukaryotic cell is a human cell.

8. A method of using a DNA segment that encodes a human nuclear protein (HEC), comprising the steps of:

a) preparing a recombinant vector in which a HEC protein or peptide-encoding DNA segment is positioned under the control of a promoter;

b) introducing said recombinant vector into a host cell;

c) culturing said host cell under conditions effective to allow expression of the encoded HEC protein or peptide; and

d) collecting said expressed HEC protein or peptide.

9. An isolated nucleic acid segment characterized as:

a) a nucleic acid segment comprising a sequence region that consists of at least 18 contiguous nucleotides that have the same sequence as, or are complementary to, 18 contiguous nucleotides of SEQ ID NO:2; or

b) a nucleic acid segment of from about 18 to about 2090 nucleotides in length that hybridizes to the nucleic acid segment of SEQ ID NO:2 or the complement thereof, under stringent hybridization conditions.

10. A method for detecting a nucleic acid sequence encoding a human nuclear protein (HEC) protein or peptide, comprising the steps of:

a) obtaining a sample nucleic acid suspected of encoding HEC protein or peptide;

b) contacting said sample nucleic acid with an isolated nucleic acid segment encoding said HEC protein under conditions effective to allow hybridization of substantially complementary nucleic acids; and

c) detecting the hybridized complementary nucleic acid so formed.

11. A nucleic acid detection kit comprising, in suitable container means, a human nuclear protein (HEC) protein-encoding nucleic acid segment and a detection agent.

12. A peptide composition comprising a human nuclear protein (HEC) that includes an 8 amino acid contiguous sequence from SEQ ID NO:1.

13. A purified antibody that binds to a human nuclear protein (HEC) or peptide.

14. A method for detecting a human nuclear protein (HEC) or peptide in a biological sample, comprising the steps of:

a) obtaining a biological sample suspected of containing a HEC protein or peptide;

b) contacting said sample with an antibody that specifically binds to said protein or peptide under conditions effective to allow the formation of complexes; and

c) detecting the complexes so formed.

15. An immunodetection kit comprising, in suitable container means, an antibody that specifically binds to human nuclear protein (HEC) or peptide, and an immunodetection reagent.

16. A composition comprising

17. A method of preparing a human nuclear protein (HEC), comprising:

a) culturing the transformed host cell of claim 5 under conditions effective to produce a HEC protein; and

b) obtaining said HEC protein from said cell.

18. A method of disordering sister chromatid alignment and separation in an interphase cell, comprising administering to said cell an inactivating amount of antibody that specifically binds to human nuclear protein (HEC) wherein mitosis is disrupted.

19. A method of modulating cell cycle progression, comprising administering to a cell an amount of human nuclear protein (HEC) effective to disrupt chromatid separation.

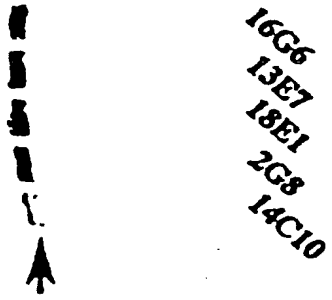

FIG. 1

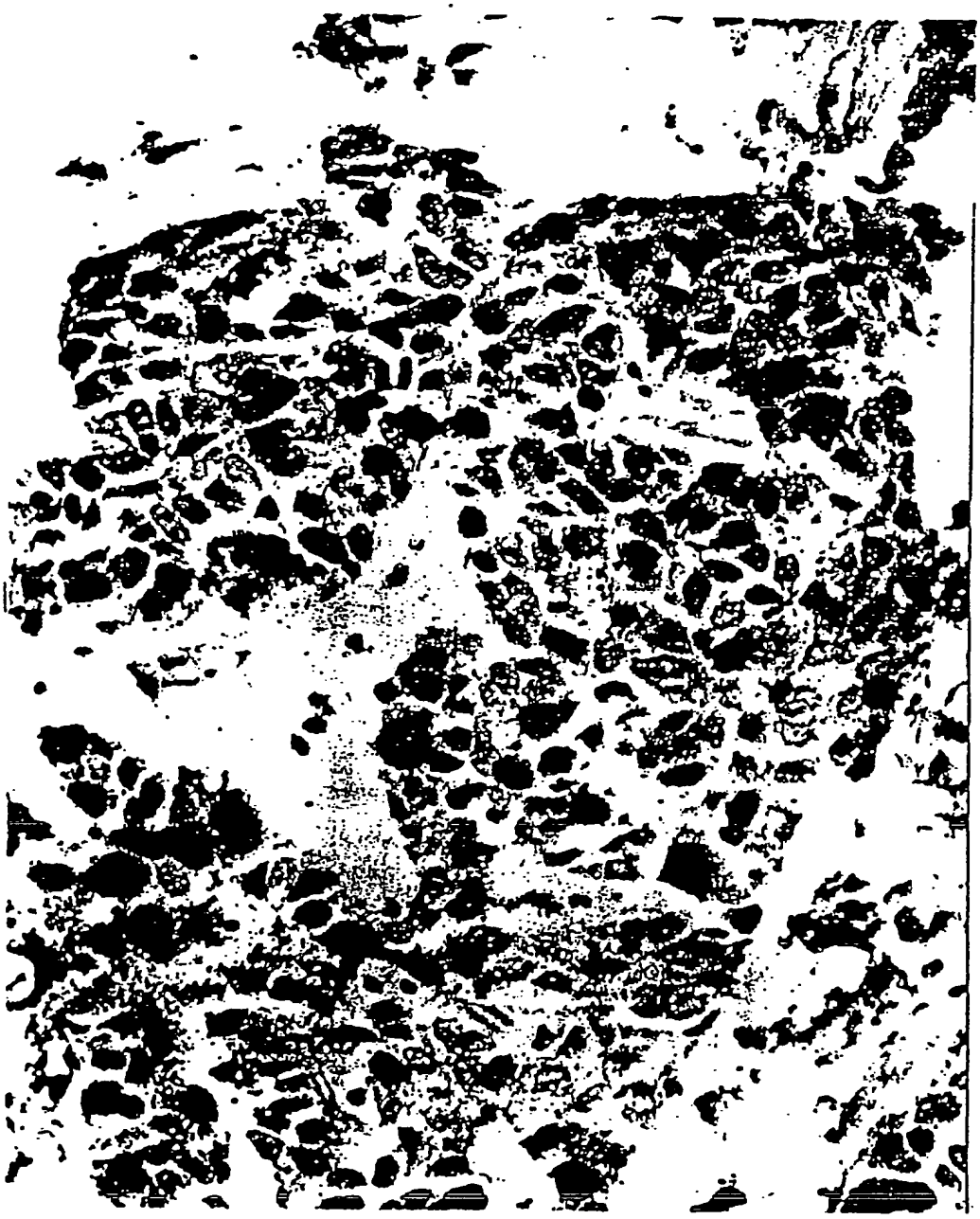

FIG. 2

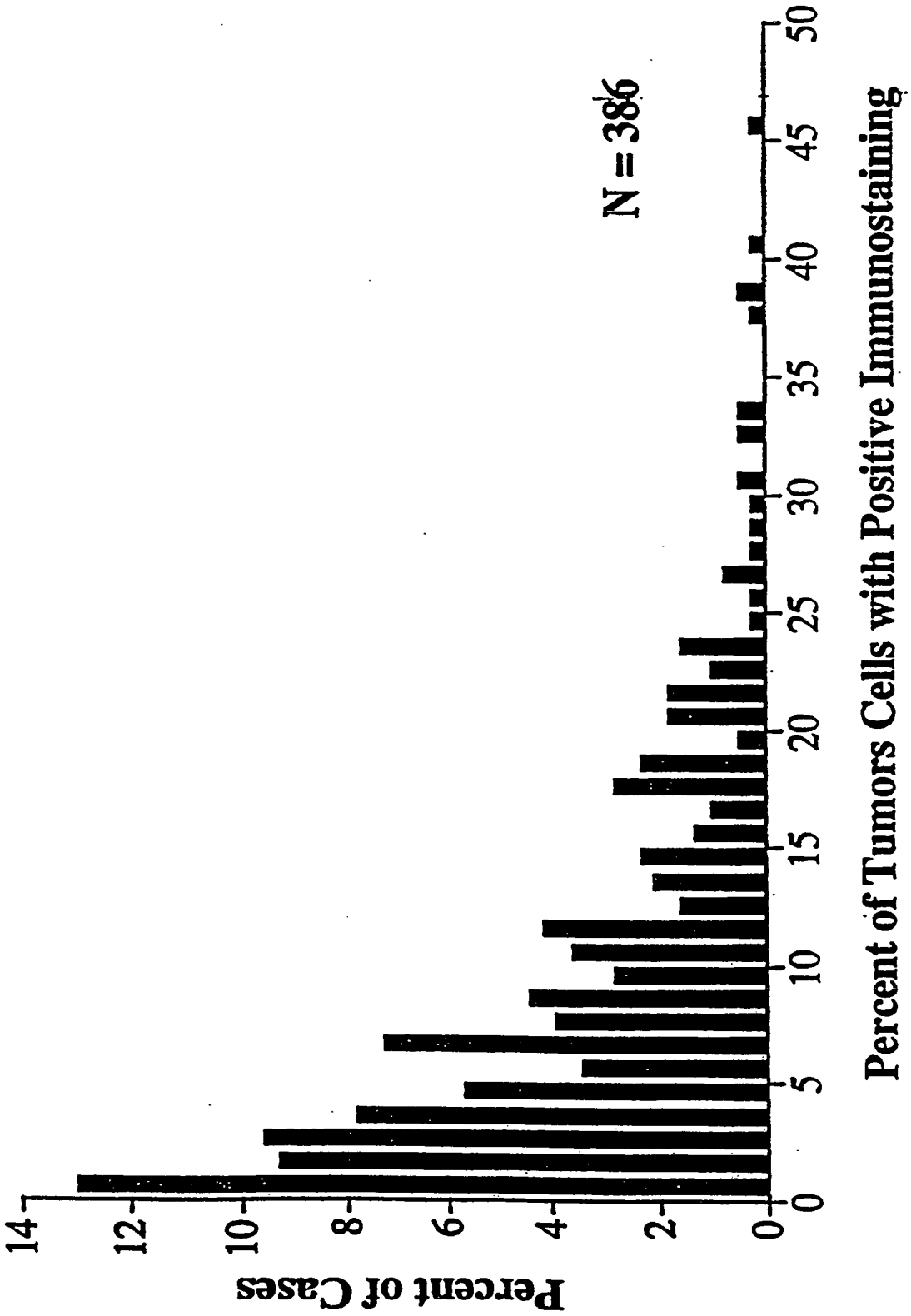
N = 386
Percent of Tumors Cells with Positive Immunostaining
Percent of Cases
0
2
4
6
8
10
12
14
0
5
10
15
20
25
30
35
40
45
50

FIG. 3

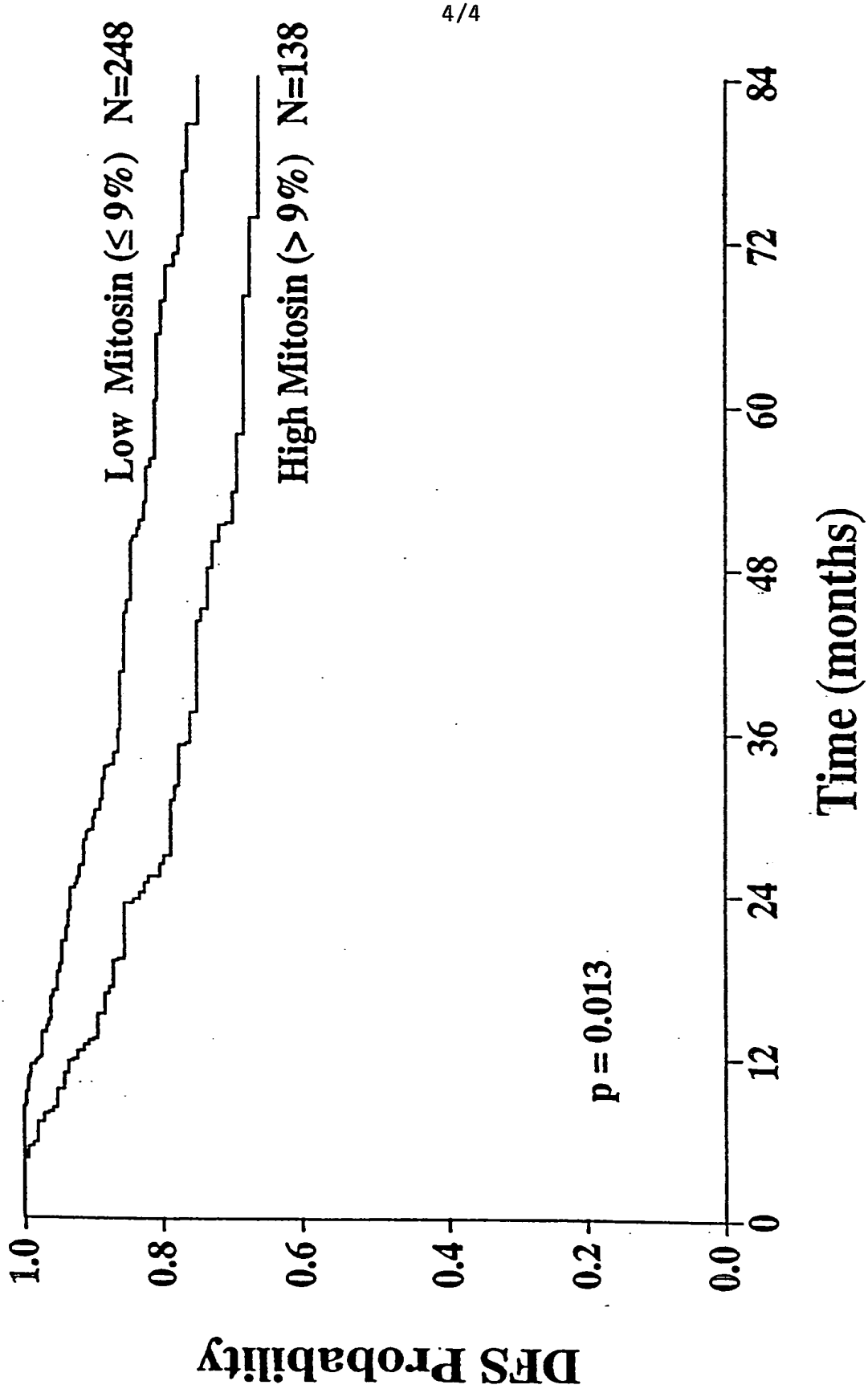

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/06727

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 A61K38/17 G01N33/50 C07K16/18

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K G01N

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HILLIER L ET AL: "Homo sapiens cDNA clone 116772 3' (ID HS46352)" EMBL SEQUENCE DATABASE, 5 April 1995, XP002068135 HEIDELBERG, GERMANY see the whole document ---	2,3,9-11
X	HILLIER L ET AL: "Homo sapiens cDNA clone 196139 5' (ID HS 253190)." EMBL SEQUENCE DATABASE, 28 August 1995, XP002068136 HEIDELBER, GERMANY see the whole document --- -/--	2,3,9-11

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicationdate of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
13 August 1998	25/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Oderwald, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COMPTON D A ET AL: "PRIMARY STRUCTURE OF NUMA, AN INTRANUCLEAR PROTEIN THAT DEFINES A NOVEL PATHWAY FOR SEGREGATION OF PROTEINS AT MITOSIS" JOURNAL OF CELL BIOLOGY, vol. 116, no. 6, March 1992, pages 1395-1408, XP000601680 see the whole document ---	
A	DURFEE T ET AL: "THE RETINOBLASTOMA PROTEIN ASSOCIATES WITH THE PROTEIN PHOSPHATASE TYPE 1 CATALYTIC SUBUNIT" GENES AND DEVELOPMENT, vol. 7, no. 4, 1 April 1993, pages 555-567, XP000571493 see abstract; figure 1 see page 556, paragraph 4 - page 557, paragraph 4 ---	
P,X	CHEN Y ET AL: "HEC, a novel nuclear protein rich in leucine heptad repeats specifically involved in mitosis." MOLECULAR AND CELLULAR BIOLOGY, (1997 OCT) 17 (10) 6049-56. JOURNAL CODE: NGY. ISSN: 0270-7306., XP002068137 see abstract; figures 2,5; table 1 see page 6050 see page 6052, paragraph 4 - page 6053, paragraph 3 ---	1-15, 17-19
P,X	CHEN Y ET AL: "HEC binds to the seventh regulatory subunit of the 26 S proteasome and modulates the proteolysis of mitotic cyclins." JOURNAL OF BIOLOGICAL CHEMISTRY, (1997 SEP 19) 272 (38) 24081-7. JOURNAL CODE: HIV. ISSN: 0021-9258., XP002068138 see abstract; figures 1,8 see page 24082 -----	1-15, 17-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 06727

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.

2. [X] Claims Nos.: 16
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
The claim does not define any matter for which protection is sought.
Claim 7: the number of the claim that it refers to is missing.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark: Although claims 18 and 19 are directed to a method of treatment of the human/animal body (as far as in vivo methods are concerned), the search has been carried out and based on the alleged effects of the compound/composition.